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International Journal of Pharmaceutics 192 (1999) 109–114

international journal of pharmaceutics

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Binding of centchroman with human serum as determined by charcoal adsorption method☆

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Received 8 March 1999; accepted 3 August 1999

Abstract

Protein binding of drugs is an important factor influencing both pharmacokinetic and pharmacodynamic parameters. Thus, knowing the extent of protein binding of drugs is crucial. Centchroman is a non-steroidal once a week oral contraceptive. It has been reported to be useful for the treatment of breast cancer and osteoporosis. Ample data has been generated on pharmacokinetics of centchroman in animals and humans. The extent of protein binding of centchroman has not been established so far. Non-specific adsorption of the drug limits the use of conventional methods like ultrafiltration and equilibrium dialysis. A method of charcoal adsorption as reported by Yuan et al. (method I) was used after modification (method II) to determine its binding to human serum. The extent of protein binding (%) is estimated from decline of percent drug remaining in the supernatant after adding the charcoal. Study was carried out at 1- and 10-μg/ml concentrations in drug free human serum samples and an HPLC assay was used to determine concentration-time data. The percentage of centchroman remaining in serum versus time data was analysed using non-linear fitting programs on WinNonlin software. Method II was found to give higher estimates of protein binding than the former method by preventing the dilution effect. Using this method, the extent of protein binding of centchroman was found to be 101.83 ± 1.28 and $94.87 \pm 3.59\%$ at 1 and 10 μg/ml, respectively. However, it was $\sim 90\%$ in the individual serum samples showing intersubject variability in protein binding of centchroman.

Keywords: Centchroman; Protein binding; Charcoal adsorption

1. Introduction

Protein binding plays an important role in pharmacokinetics and pharmacotherapeutics. It influences the distributional, pharmacological, and pharmacokinetic properties of drugs (Kwong, 1985). It is often determined by equilibrium dialysis, ultrafiltration or ultracentrifugation. These

☆ CDRI communication # 5856

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0378-5173/99/$ - see front matter
PII: S0378-5173(99)00304-X

methods operate under equilibrium conditions and are based on the separation of free drug from the bound drug under the same conditions (Kurz et al., 1977), and have their own merits and demerits. These methods are not useful for compounds exhibiting extensive non-specific adsorption, or have apparent lipid partitioning into low and very low density lipoproteins (Yuan et al., 1995).

Centchroman (ormeloxifene hydrochloride) (CC) [3,4-*trans*-2,2-dimethyl-3-phenyl-4(*p*-β-pyrrolidino-ethoxyphenyl) -7-methoxy chroman] is a non-steroidal once a week oral contraceptive developed by the Central Drug Research Institute, Lucknow. CC is officially cited in Indian Pharmacopoeia (Indian Pharmacopoeia, 1996) and is marketed under the trade names Saheli and Centron. It has been found to be useful for the treatment of breast cancer (Misra et al., 1989) and may be therapeutically effective for other clinical conditions such as dermatitis (Piggott, 1995), osteoporosis (Labroo et al., 1995) restenosis, endometriosis and uterine fibroid disease (Bryant and Dodge, 1995).

CC has low affinity, non-saturable and reversible binding with a protein similar to albumin in plasma of rhesus monkey (Agnihotri et al., 1996). Binding of CC with plasma proteins of rat, rabbit, guinea pig and rhesus monkey has also been reported (Ratna et al., 1991). Due to its extensive non-specific adsorption on dialysis cell, ultrafiltration devices and their membranes, the conventional methods for protein binding determination could not be used. Therefore, a novel charcoal adsorption method reported by Yuan et al. was used after suitable modifications (Yuan et al., 1995). The modifications were necessary to minimize the effect of dilution on protein binding and to make the method suitable for the drug in question. This method operates under non-equilibrium conditions and involves measuring the time course of decline of the concentration of bound drug when the free drug is being adsorbed and removed by dextran coated charcoal. The extent of protein binding (%) is estimated from decline of percent drug remaining in the supernatant after adding the charcoal. The method is fast, easy and versatile, and can be used for determination of protein binding of similar drugs in different binding matrices like plasma, serum and pure protein solutions.

2. Materials and methods

Centchroman [CC] pure reference standard was synthesized and purified (> 99%) in this institute. Charcoal granular 4–14 mesh and dextran (avg. mol. wt. 71 200) were procured from Sigma, USA. Dulbecco's phosphate buffered saline (DPBS) (Ca and Mg free) was procured from HiMedia Laboratories, Mumbai, India. Potassium dihydrogen orthophosphate AR grade was procured from S.D. Fine-Chemicals, Bombay, India. Orthophosphoric acid AR grade was supplied by Qualigens Fine Chemicals, Mumbai, India. HPLC grade acetonitrile was procured from Ranbaxy Laboratories, Chemical Division, India. Water was used after triple distillation through all-quartz water distillation apparatus. Normal drug free human serum (NHS) was separated from the blood of healthy subjects and used either as such or after pooling.

The dextran coated charcoal suspension was prepared by adding 0.6 gm of charcoal to 100 ml of DPBS containing 0.06% dextran. The mixture was stirred with a magnetic stirrer at room temperature until the charcoal was suspended. This suspension was prepared at least 18 h before use and stored at 5–10°C for not longer than 30 days. The stored charcoal mixture was resuspended before use (Yuan et al., 1995).

The binding was determined by methods I and II in pooled and individual serum (subjects 1 and 2) as described below.

The study was carried out in duplicate sets at 1- and 10-μg/ml concentration levels of CC. The ratio of binding matrix and charcoal suspension was 1.66:1. NHS was spiked with CC and allowed to equilibrate for 10 min before the study. Spiked NHS (5 ml) was transferred into a 30-ml glass tube immersed in an oil bath maintained at 37 ± 1°C and mounted on a magnetic stirrer. With continuous stirring 3 ml of charcoal suspension was added to serum. Serial samples (375 μl) were withdrawn from this continuously stirred mixture

at 0.5, 1, 2, 4, 8, 15, 30, 45, 60, 90, 120, 180 and 240 min into 0.6-ml polypropylene microcentrifuge tubes and centrifuged immediately at 11 000 rpm ($\sim 15\,000 \times g$) at 37°C using a refrigerated microcentrifuge. Aliquots (100 μl in triplicate) of supernatant from each tube were immediately transferred into 3-ml glass centrifuge tubes and stored at −30°C until analyzed for drug content by HPLC (referred to as method I in the study).

To overcome the dilution effect due to charcoal suspension, method I was modified. First 3 ml charcoal suspension was transferred into a 30-ml glass tube, centrifuged (at $2000 \times g$) for 10 min at 25°C, and the supernatant DPBS was carefully decanted off, and spiked NHS (5 ml) was added to the charcoal pellet under continuous stirring at 37 ± 1°C. Serial samples (375 μl) were withdrawn from stirred mixture between 0.5 and 240 min as described in method I, and supernatant was separated and stored in the manner described in method I. This study was carried out in duplicate sets in NHS at 1-μg/ml concentration of CC.

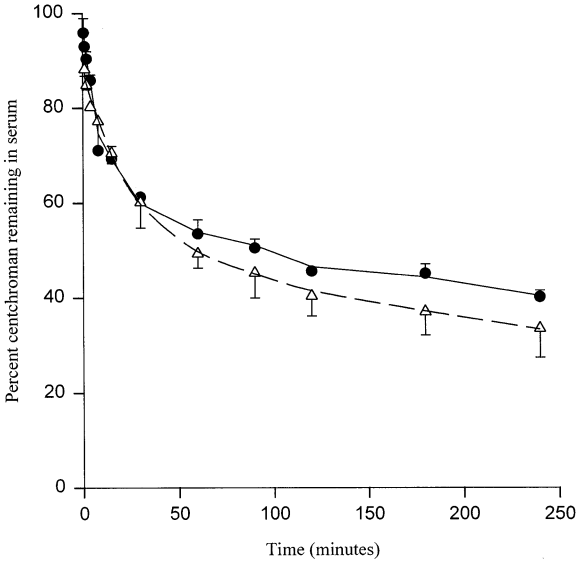

Fig. 1. Percentage of centchroman remaining in fortified pooled human serum after adding charcoal suspension at 1- and 10-μg/ml concentrations using method I. Each symbol represents mean ± S.D. of $n = 6$. ●, Observed (1 μg/ml); — predicted (1 μg/ml); △, observed (10 μg/ml); ---, predicted (10 μg/ml).

As a further modification, the above study was repeated with 6 ml of charcoal suspension (twice the amount used in method I) in the same manner except that sampling was done until 120 min. This study was carried out in triplicate sets at 1- and 10-μg/ml and in duplicate sets at 5-μg/ml concentration of CC in NHS (referred to as method II in the study).

The aliquots (100 μl) of serum samples from methods I and II were processed by protein precipitation method and CC concentrations were determined by the HPLC assay method of Paliwal and Gupta (1995).

Percent drug remaining in the supernatant NHS versus time data was fitted to a two-compartment model with i.v. bolus input using a non-linear regression programme on WinNonlin software (Version 1.5). This model is equivalent to that described by Yuan et al. for determination of protein binding by charcoal adsorption method. The model is described by the following biexponential equation:

$$B(t) = A_1 e^{-\alpha t} + A_2 e^{-\beta t}$$

where $B(t)$ is % bound at time t, A_1 and A_2 are Y intercepts, and α and β are distribution and disposition rate constants for the two phases, respectively. The extent of protein binding was given by the C_{max} value (at time $t = 0$). Association (K_a) and dissociation (K_d) rate constant of CC with serum proteins were calculated by k_{12}, and k_{21}, respectively in the WinNonlin output data. Protein binding data and K_a, K_d values obtained by the two methods, at two concentration levels were compared by applying t-test and t-test with Behrens-Fisher procedure (Bolton, 1997).

3. Results

Figs. 1 and 2 show % decline of CC in NHS at 1- and 10-μg/ml concentrations after adding charcoal with methods I and II, respectively. The best fit parameters according to two-compartment open model with bolus input of WinNonlin for methods I and II at 1-, 5- and 10-μg/ml concentrations are given in Table 1. The estimates of %

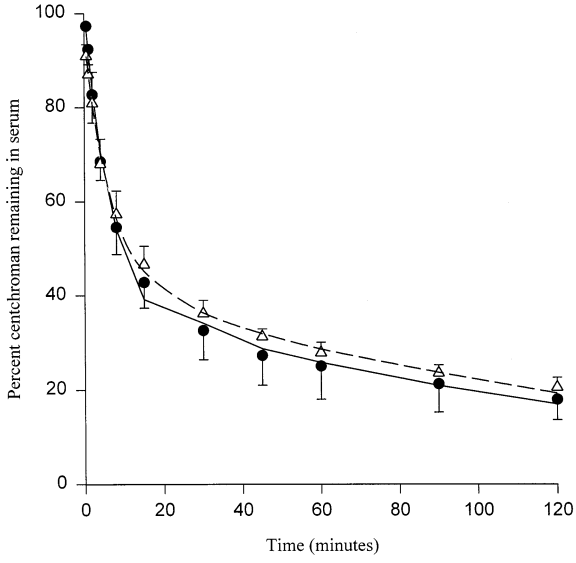

Fig. 2. Percentage of centchroman remaining in fortified pooled human serum after adding to charcoal at 1- and 10-μg/ml concentrations using method II. Each symbol represents mean ± S.D. of $n = 6$. ●, Observed (1 μg/ml); —, predicted (1 μg/ml); △, observed (10 μg/ml); ---, predicted (10 μg/ml).

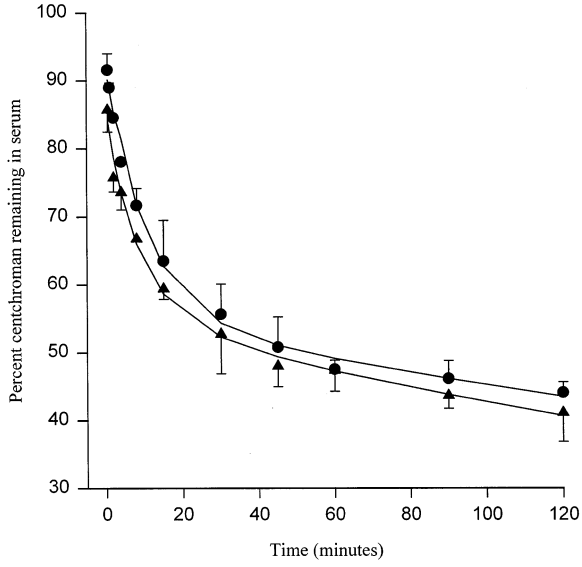

Fig. 3. Percentage of centchroman remaining in fortified serum of subjects 1 and 2 after adding to charcoal at 5-μg/ml concentration using method II. Each symbol represents mean ± S.D. of $n = 4$. ●, Observed (subject 1); —, predicted (subject 1); ▲, observed (subject 2); —, predicted (subject 2).

CC bound in NHS at 1- and 10-μg/ml levels were higher with method II than method I. However, the estimated % bound was significantly less at 10-μg/ml level than 1 μg/ml by both methods ($P < 0.05$) showing non-linearity in protein binding.

Protein binding results of CC in individual NHS samples of subject 1 and subject 2 at 5-μg/ml level by method II are given in Table 1. The protein binding was found to be 87.15 ± 2.65 and 91.91 ± 1.59% in two subjects. Fig. 3 shows the percent decline of centchroman in serum of two subjects at 5 μg/ml. The total protein content in the pooled and individual serum samples was estimated by the method of Lowry et al. (1951). Fig. 4 shows the effect of total protein content on binding of CC in NHS. The % binding of CC in NHS was found to increase with the total protein content of serum samples.

Table 1
WinNonlin output parameters (mean ± S.D., $n = 6$) obtained at CC concentrations of 1, 5 and 10 μg/ml by methods I and II in different serum samples

Concentration (μg/ml)	Serum sample	Method	% Bound	k_{12} (K_a) [(min^{-1}) $(\mu g/ml)^{-1}$]	k_{21} (K_d) [$(min^{-1})(\mu g/ml)^{-1}$]
1	Pooled	I	95.18 ± 1.05	0.0283 ± 0.0008	0.0483 ± 0.0071
1	Pooled	II	101.83 ± 1.28	0.0856 ± 0.0136	0.0673 ± 0.0011
10	Pooled	I	88.16 ± 2.08	0.0292 ± 0.0075	0.0422 ± 0.0106
10	Pooled	II	94.87 ± 3.59	0.0736 ± 0.0190	0.0766 ± 0.0161
5[a]	Subject 1	II	87.15 ± 2.65	0.082 ± 0.029	0.113 ± 0.001
5[a]	Subject 2	II	91.91 ± 1.59	0.035 ± 0.009	0.055 ± 0.005

[a] Mean ± S.D., $n = 4$.

CC was found to adsorb non-specifically to the construction material of dialysis cell and membrane when conventional methods such as equilibrium dialysis and ultrafiltration were used. Percent drug loss on incubating the fortified buffer for 4 h at 37°C in dialysis cell, with and without membrane, was 99.56 ± 0.58 ($n = 3$) and 89.53 ± 0.55 ($n = 3$), respectively. Due to non-specific adsorption of CC, equilibrium between serum and buffer compartment could not be achieved even up to 8 h. Ultrafiltration, using Centrifree micropartition system (Amicon, Baverly, MA, USA), was found to adsorb CC on to the tubes and cups of Centrifree devices to an extent of $46.46 \pm 4\%$ and $64.73 \pm 1.2\%$ ($n = 3$), respectively, and no drug passed through the YMT ultrafiltration membrane. Therefore, the alternative method of charcoal adsorption was used.

The charcoal adsorption method for determining the extent of protein binding is based on the continuous removal of unbound drug over a time period and determination of % bound drug at each time point. Association and dissociation of drug with proteins is a dynamic phenomena and charcoal acts as a sink for the removal of free drug. This method is widely used to separate free and bound ligand in radioimmunoassays as compared to determination of extent of protein binding. The direct use of this method for protein binding studies give underestimates because: (a) dilution of protein solution occurs upon charcoal addition; (b) K_a for protein binding is much lower than the K_a for antigen-antibody interactions; and (c) drug-protein interaction is a dynamic phenomena and bound drug dissociates from proteins while the free drug is removed continuously by charcoal. The kinetic approach applied in the method reported by Yuan et al. and in the present study takes into account the dynamic feature of protein binding. In fact it takes advantage of this feature where only percent bound is determined at each time point and used to estimate the maximum binding possible at time $t = 0$.

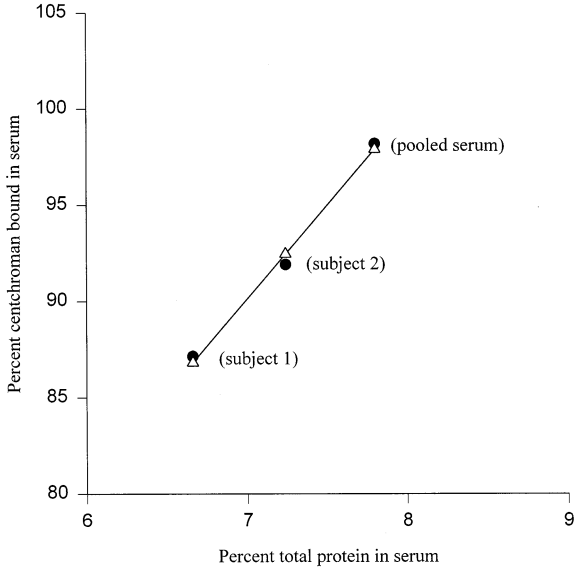

Fig. 4. Effect of total protein content on protein binding of centchroman. ●, Observed value; △, fitted value.

The effect of dilution on protein binding estimation is evident from the difference in estimates of percent CC bound as determined by methods I and II (Table 1). With method I, which involves dilution of serum, the protein binding was found to be 95.18 ± 1.05 and $88.16 \pm 2.08\%$ at 1 and 10 μg/ml, respectively. Whereas method II, which is devoid of any dilution, resulted in higher protein binding estimates of 101.83 ± 1.28 and $94.87 \pm 3.59\%$ at 1 and 10 μg/ml, respectively. Dilution effect has been reported with rhesus monkey plasma where 50% dilution decreases the plasma protein binding of CC from 80 to 40% (Agnihotri et al., 1996). Yuan et al. also underestimated the protein binding of SC-52151 due to dilution resulting from addition of charcoal suspension. The present study (method II) takes care of the dilution effect. Although, the initial experiments with 3 ml of charcoal suspension gave variable estimates of % bound (92.3 and 83.21), k_{12} (0.046 and 0.032) and k_{21} (0.043 and 0.019), use of twice the amount of charcoal in the method gave better and consistent estimates of percent bound value in relatively a short span of time (120 min as compared to 240 min in method I and first modification). It was observed that for CC method II (Fig. 2) was devoid of fluctuations as compared to method I (Fig. 1) at individual time points. It may be due to insufficient amount of charcoal used in method I for the removal of drug in question

from NHS. Hence, by increasing the amount of charcoal, without diluting the binding matrix, it was possible to achieve a reliable and reproducible method for estimation of protein binding of CC.

The k_{12} and k_{21} values obtained by methods I and II at 1- and 10-μg/ml levels were comparable ($P > 0.05$) (Table 1). This shows that methods are consistent as far as kinetics of drug-protein interaction is concerned. However, k_{21} was greater than k_{12} in case of method I at both concentrations, showing that dissociation was higher than that indicated by method II where k_{12} was greater than k_{21}. It may be due to dilution of binding matrix after addition of charcoal suspension and the same effect may account for the underestimation of % bound value for CC with method I.

CC was found to exhibit a great inter-subject variability in protein binding in NHS of two human subjects as determined at 5-μg/ml level (Fig. 3). The protein binding was found to be exceptionally lower than that obtained at 10-μg/ml level in pooled NHS. The difference in total protein content of the serum samples used in the study was thought to be responsible for this. The percent binding of CC was correlated with total protein content among the serum samples used in the study. The % binding increased with an increase in total protein content (Fig. 4). This shows that CC may have a saturation in binding at concentrations above 1 μg/ml.

To conclude, the modified charcoal adsorption method gives a better and closer estimate of protein binding of CC. Using this method the protein binding of CC was found to be 101.83 ± 1.28 and 94.87 ± 3.59% at 1 and 10 μg/ml, respectively. It overcomes the problem of non-specific adsorption encountered in conventional methods (Kurz et al., 1977) and that of dilution of binding matrix encountered in reported method (Yuan et al., 1995). The method is fast, easy, reliable and requires a simple experimental set-up.

Acknowledgements

Authors thank C.M. Gupta, Director of this institute for providing the facilities. Financial assistance to two of us (V.P.K. and M.K.) by the Council of Scientific and Industrial Research, India (Emeritus Scientist project # 21(410)/97/EMR-II) is gratefully acknowledged.

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